1962

An Experimental Analysis and Description of the Melanocytes in the Leg Ofpet Mice.

Thomas Carl Mayer
Louisiana State University and Agricultural & Mechanical College

AN EXPERIMENTAL ANALYSIS AND DESCRIPTION OF THE MELANOCYTES IN THE LEG OF *PET* MICE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Zoology, Physiology
and Entomology

by
Thomas Carl Mayer
B.A., University of Tennessee, 1953
M.S., The Johns Hopkins University, 1957
June, 1962

ACKNOWLEDGMENT

I am indebted to Dr. Willie M. Reams, Jr. for his technical assistance, enlightening discussions, and encouragement during the course of this investigation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ILLUSTRATIONS

ABSTRACT

The presence of melanocytes located consistently within certain muscles of the leg of PET mice was examined experimentally in order to determine the embryogenesis of this condition. The origin of these extra-epidermal melanocytes was investigated by grafting various regions of the leg of mouse embryos into the coelom of White Leghorn chick embryo hosts. In this manner the migration of melanoblasts in the mouse embryo was traced from their origin in the neural crest to the dorsal skin of the leg where they appeared first by 12 days of gestation. The ventral surface of the leg was populated during the following day of development, and two days later the mesodermal tissue of the leg was penetrated by melanoblasts. In contrast to this sequence of appearance of melanoblasts, pigment-containing cells were found in the musculature of the leg by the 17th day of gestation, and their number increased until the mouse was one week old, at which time the adult complement of 2,100 melanocytes was reached. Evidence is presented which indicates that the adult number of pigment cells was attained through proliferation of melanoblasts arising from the initial colonization of the mesoderm, and not through a continuous migration from the skin.

A study of 8 strains of pigmented mice revealed melanocytes to be present within the musculature of these forms, although never

in as large a number as in the PET strain. Thus it seemed that the tissue environment in the PET strain does not differ basically from that of other mice in supporting melanogenesis. Results of skin grafting experiments between leg and belly skin of newborn, and fetal and newborn, PET mice placed the primary responsibility for observed regional pigmentary variations on a differential migration of melanoblasts into these regions. The possibility of amelanotic melanocytes being present in the unpigmented skin and muscles of the leg of PET mice was doubted.

The mutual repulsion of pigment cells as described by Twitty could well be the mechanism accounting for the appearance of melanocytes within the musculature. Continued proliferation of pigment cells following complete saturation of the skin would result in the penetration of the deeper mesodermal tissues by melanoblasts, where they produce melanin. The varying degrees of pigmentation in the strains studied would thus be an expression of the proliferative capacity of the melanoblasts.

I. INTRODUCTION

Pigmented mammals generally have been described as possessing melanocytes associated with the skin and its derivatives, and with the choroid, the retina, and the iris of the eye. On occasion, melanocytes have been reported to occur in extra-epidermal regions as well, although not with any degree of predictability from one individual to the next. Branched melanin-containing cells have been observed with some regularity in the connective tissue of the parathyroid glands of sheep (Schaper, 1895), mice (Dunn, '49), and the gray rat (Addison and Fraser, '32). The hypophysis of the latter also possessed melanocytes, with the intermediate lobe being especially rich in pigmentation. Other extra-epidermal tissues in which melanocytes occur in the mouse are the meninges, especially between the olfactory and cerebral hemispheres; the thymus, and harderian gland (Markert and Silvers, '56). The adrenal medulla and lymph nodes in man are sometimes pigmented (Masson, '48), as are the adrenal capsule and the walls of the pelvic blood vessels of sheep (Grant, '33). Certain marsupials are characterized by possessing pigment within the reproductive tract (Burns, '39), and a similar association may exist in the placental mammals (Billingham and Silvers, '60).

Even in the skin considerable variability in melanocyte occurrence has been observed among various forms. In the mouse melanocytes are restricted to the hair follicles, and are lacking in the dermis and epidermis generally. However, in the relatively hairless regions of the body such as the nose, ear, soles and scrotum, both the epidermis and dermis are rich in melanocytes. There is only one report of melanocytes occurring, although in very small numbers, in the dermis of other regions of the mouse (Steiner-Wourlisch, '25). In the newborn mouse the epidermis is pigmented throughout, but by the 12th to 14th days of age melanocytes no longer are found in the epidermis and are restricted to the hair follicles (Rawles, '55). The condition in the mouse is true also for the rat and rabbit. However, during hair formation in the guinea pig melanocytes do not disappear from the epidermis but remain throughout life (Billingham, '48). It has been reported that melanocytes are more widely distributed in the young guinea pig epidermis than in the adult (Bischitz and Snell, '59). In man melanocytes are found in the epidermis in all regions of the adult body, and have been the subject of extensive investigation. Dermal pigmentation in man is characteristic of the fetus, and may remain in the form of Mongolian spots in the young. However, dermal pigmentation seldom persists in the adult human (Zimmermann and Becker, '59). Adult primates as the chimpanzee, orangutan, baboon, barbary ape and macacus are regularly pigmented in the dermis (Adachi, '03).

The most common explanation for the above differences in pigmentation has been that a particular region in one form is capable

of sustaining melanogenesis, while in the same region of another form melanin synthesis cannot occur. For example, it commonly is supposed that the superficial epidermis of the adult mouse is an unfavorable locus for melanogenesis, and pigment cells, although present in the area, are free of melanin. On the other hand, in the region of the snout, ears and tail, the epidermis apparently presents a more favorable site for melanin synthesis, and pigment cells in these regions contain dark granules scattered throughout their cytoplasm. It should be recognized, however, that another possibility is likely as an explanation for these regional differences in pigmentation. Melanoblasts may actually be absent from the epidermis of the mouse except in the relatively hairless regions of the body, where they appear as melanocytes. The reported attraction of melanoblasts to the hair follicles could well account for the different locations of melanocytes in these regions. According to this analysis, the variation between guinea pigs and mice could simply be due to a greater production of melanoblasts in the former animal, which results in melanocytes appearing between the hair follicles in the epidermis. Variations in the time and extent of migration of melanoblasts from the neural crest is not uncommon, and has been held responsible for differences in pigmentary patterns in various animals, as has been shown by Twitty ('44) in amphibia, and Auerbach ('54) in mice.

In 1958 a strain of mice was established at the Medical College of Virginia which was considered to have an unusual pigmentary pattern

in the skin and extra-epidermal areas. Termed the PET/MCV strain (Pigmented Extra-epidermal Tissues), these mice were originally described as having pigment cells consistently distributed in the connective tissue of the pleura, diaphragm, and peritoneum (Reams and Nichols, '59). A subsequent more detailed analysis of the tissues of this strain revealed melanocytes to be almost ubiquitous in distribution, and were consistently absent only from the connective tissue of the gut lining (Nichols and Reams, '60). Sites noted to be particularly rich in melanin-producing cells were the skin, connective tissue of the lungs, kidney, ribs and cartilages, gonads, intercostal and extremity muscles, semicircular canals, and other tissues. The distribution of melanocytes was not, however, consistent from litter to litter, or even within members of the same litter. Since the establishment of the original strain, a substrain showing uniformity in the appearance of melanocytes in the skin and musculature of the hind limb has been isolated and maintained. In a preliminary report on this PET/LSU substrain, Reams and Mayer ('61) described extra-epidermal melanocytes as being limited primarily to the muscles of the posterior and lateral compartments of the leg. Mice of this substrain show a high predictability in the occurrence of melanocytes in the musculature, and offer an opportunity for the study of factors responsible for the migration and maturation of pigment cells in an extra-epidermal environment.

The neural crest origin of epidermal pigment cells in mammals is known from the work of Rawles ('40, '47). Neural crest free prospective skin from early mouse embryos grafted into the coelom of White Leghorn chick embryos produced skin and hair that were devoid of pigment cells. Grafts of similar tissue from older mice into which the neural crest had already migrated produced pigmented hair and skin. Using this method Rawles was able to map out generally the medio-lateral spread of melanoblasts from their neural crest origin, and demonstrated that by the end of 12 days of embryonic development melanoblasts were present in all the major regions of the body. This study by Rawles is the only direct evidence available for mammals relating to the neural crest origin of melanocytes, and their migration throughout the embryo. Some investigators have used histochemical methods to detect precursor pigment cells and follow their routes of migration. Danneel and Cleffmann ('54) employed the silver nitrate reaction to reveal melanoblasts. They first observed branched cells in mice at the dermal-hypodermal junction of the snout by 14-15 days of gestation, and followed their subsequent appearance in the epidermis and then the hair follicles. The suggestion was offered that this sequence of melanoblast appearance is evidence of migration in this direction. However, in view of the early dispersal of melanoblasts in the mouse as shown by Rawles, and the lack of suitable staining methods to detect these cells during their early developmental stages, one must use caution in interpreting melanoblast maturation

as evidence of migratory sequence. Reliance should be placed when possible on grafting methods to reveal the presence of melanoblasts in the tissues in question.

The purpose of this investigation is, therefore, to examine the distribution of pigment cells within the muscles of the PET mouse; to determine by grafting methods the origin and migratory pathway of melanoblasts into this extra-epidermal location; and to investigate the factors responsible for the appearance of pigment cells within the musculature.

II. MATERIALS AND METHODS

Mice used in the present study were primarily of the inbred PET/LSU strain maintained at Louisiana State University. In addition, mice of 11 other strains were employed in a comparative study to be discussed later. Pregnancies were timed by placing a male with a female in estrus during the evening, and examining the female the following morning for a vaginal plug. Embryos used in the descriptive phase of this study were obtained by sacrificing the female, removing the embryos from the uterus, dissecting them free from the membranes, and fixing them in 5% formol-saline. This fixative was selected in anticipation of staining some of the embryos with ammoniacal silver nitrate. The exact developmental age of the embryos was determined by morphological criteria, since it was observed that members of the same litter often varied in development. Features that were found of value in staging were overall length, the pigmentation of the eye, closure of the eyelid, and contour of the limbs. Since little information was available on the developmental stages of mouse embryos of the ages used in this study, some embryos of each litter were set aside to construct a normal staging series. Embryos to be examined for pigmentation were fixed, hemisected, dehydrated, cleared in oil of wintergreen, and the individual muscles dissected from the leg. Each muscle

was isolated, mounted on a slide in oil of wintergreen, and examined for the presence of melanocytes. Counts were made with the aid of a grid ocular micrometer. Ages used in the descriptive study were from 16 days after fertilization to one week postnatal, two weeks, one month, and one year (adult). The lateral head of the gastrocnemius, one of the more heavily pigmented muscles of the leg, was sectioned and lightly stained with Delafield's hematoxylin in order to determine the exact location of the pigment cells with respect to the muscle fibers.

In the experimental stage of this study, mouse embryos used as donors ranged in age from 11½ to 17 days of gestation. The developmental age of the embryo was determined in the manner described previously. In this series, however, the female mouse was anesthetized with nembutal during the course of the operations. Embryos then could be removed one at a time and the donor tissue prepared for grafting while the littermates remained within the uterus of the female. In the preparation of the tissue for transplantation, the mouse embryo with its attached membranes was transferred to a dish of warm, sterile saline solution, and then removed from its membranes. The leg was detached from the embryo and the region to be grafted was isolated and placed in an operating dish containing warm chick amniotic fluid. This region was then divided into pieces 0.2 mm square, and each piece grafted into the coelom of White Leghorn chick embryo hosts. Individual muscles could be dissected from mouse embryos of ages 15 days and later. At earlier stages the

ectoderm was stripped from the limb while still attached to the embryo, and the limb isolated and split into dorsal and ventral halves. These portions were then divided into square fragments and transplanted into the chick. At ages of 14 days and earlier the hind limb is of paddle shape, and its dorsal surface corresponds to the future anterior surface of the adult. When mesodermal tissue alone was used for the transplantations, a few grafts consisting of ectoderm and mesoderm were also made, and these cases served as controls. Ectoderm alone was used in a group of transplantations. Usually more embryos were present in a litter than could be utilized for grafting during one sitting. Those remaining were preserved for histological study.

It may be noted that the ectoderm can be stripped off the leg at early ages without great disturbance to the underlying prospective dermis. Certainly a few mesodermal cells adhere to the removed ectoderm, but these cells are all superficial, lying immediately subjacent to the ectoderm.

Fertile White Leghorn eggs, obtained from the LSU poultry farm, were incubated at 38° C for 60-65 hours to give embryo hosts of H. and H. stage 17. The egg, after candling to determine the exact location of the embryo, was placed on a cotton pad in a Syracuse watchglass to facilitate handling. A rectangular piece of shell one centimeter square was sawed directly over the embryo and removed. The exposed shell membrane was then moistened with saline, and the

air space punctured to lower the embryo. The shell membrane was carefully removed thus exposing the embryo. In most cases the amnion had not formed over the posterior portion of the embryo, and did not have to be disturbed. A small slit was made on the side of the embryo through the somatopleure immediately anterior to the limb bud, and this incision served as an entrance to the coelom. The donor tissue was transferred to the operated area, inserted into the coelom, and gently pushed slightly posteriorly away from the slit in the body wall. The original piece of shell was returned to the window of the egg, sealed with paraffin, and the egg returned to the incubator. Generally grafts were recovered after 12 days of incubation, when their age corresponded to that of a 5-day mouse, a time when pigmentation is well established in the musculature of the hind limb. For recovery of the grafts, hosts were fixed in 5% formol-saline, hemisected, dehydrated, and cleared in oil of wintergreen. Examination of the coelom of the host usually revealed the graft attached to the parietal wall, and only seldom to the mesenteries. All grafts were examined in situ for visible pigment cells, and some were selected for staining and histological examination.

Homoplastic and autoplastic skin transplantations between newborn, and fetal and newborn PET mice were also undertaken in this study. In order to test the effect of environmental influences on melanocyte expression, exchanges were made between posterior leg skin and belly skin. In the case of autoplastic exchanges, newborn mice were anesthetized by chilling them in crushed ice for a few

minutes, and then 2.0 mm squares of skin were removed from the posterior surface of the leg, and from the belly, and exchanged. The grafts were held in place by covering the area with a piece of dialysis cellophane moistened with saline and cut slightly larger than the operated area. The entire region was covered with a small piece of thin adhesive tape, which was allowed to remain about 24 hours until the graft was healed. A similar procedure was followed in the homoplastic transplantations, although in this series fetal mice of 16 days age were used as donors. Host animals were newborn mice, and the regions used in the operations were the same as in the autoplastic series. Grafts were permitted to grow 5 to 6 weeks,and then were recovered by shaving the operated leg and belly skin, removing the region to be studied, and fixing in 5% formol-saline. Grafts were examined in toto for melanocytes after clearing in oil of wintergreen, and some were studied histologically.

III. NORMAL STAGES IN THE DEVELOPMENT OF PET MOUSE EMBRYOS

Since the present study is dependent on a relatively accurate staging of the donor embryos, and in view of the dearth of information available on the normal stages of the later development of the mouse embryo, a study was made of the distinguishing characteristics of PET embryos at established ages. Rawles ('47) in her work on the neural crest of the mouse relied largely on somite counts to stage embryos between 8 and 11 days of age. After 11 days, however, somite counts are impossible and other criteria must be used as an index of developmental age. Although Rawles mentioned that features such as crown-rump length, and the development of the limbs were of value in staging embryos, she did not publish the details of the sequence. Gruneberg's ('43) report on hybrid mice is the only description of the normal stages in the development of mouse embryos during the latter half of embryonic life. For the purpose of quick identification of the age of PET embryos under the operative conditions of this study, other criteria were found of more assistance in staging than those listed by Gruneberg. A brief description of these features is therefore included in this report.

Matings were timed as follows: a male was placed with a female in estrus in the evening, and removed the following morning,

at which time the female was examined for a vaginal plug. The time of gestation was counted from when the male was removed from the cage. Since mating occurs most frequently during the first half of the night (Snell, Feteke, Hummel and Law, '40), it was assumed that the timing of litters averaged about 8 hours younger than their actual age.

Crown-rump length was a less reliable index of development than many other staging features, due mainly to the manipulation of the embryos when they were removed from the uterus in early periods (up to 13 days), and to contraction and stretching when placed in fixative at later stages. During the 11th, 12th and 13th days the degree of pigmentation of the eye was an obvious feature of the developing embryo. The closure of the eyelid was a valuable index of age during the 14th, 15th and 16th days, and the development of whiskers and hair was used in staging during the last few days of prenatal life. The degree of development of the limbs was a diagnostic feature from day 9 to the 17th day of gestation, and was one of the most important indexes of age.

The following description is designed as a concise summary of changes of features of value in staging PET mouse embryos from the 10th to the 17th day of gestation.

10½ days: Crown-rump length 3.4 to 4.5 mm
Pigmentation lacking in the eye
Anterior limb bud semicircular in outline

11½ days: Crown-rump length 5.0 to 5.4 mm
Pigmentation reaching equator of eye
Anterior footplate circular in outline

12½ days: Crown-rump length 7.0 to 8.4 mm
Pigmentation reaching choroid fissure of eye
Anterior footplate clearly indented

13½ days: Crown-rump length 8.0 to 9.6 mm
Fingers on front legs separating distally
Lower eyelid covering a small portion of eye

14½ days: Crown-rump length 10.5 to 11.5 mm
Fingers on front legs separate distally,
slight webbing proximally
Lower eyelid approaching equator of eyeball

15½ days: Crown-rump length 13.5 to 14.5 mm
Slit remaining of the opening in eyelid
Fingers separate and phalanges begin to show

16½ days: Crown-rump length 15.4 to 16.7 mm
Whiskers first protruding from the follicles
Eyelids completely fused

IV. DISTRIBUTION OF MELANOCYTES WITHIN THE LEG OF PET MICE

Prior to the establishment of the PET strain, melanocytes had been observed to occur consistently only in the hair follicles of pigmented mice, and in the other limited areas previously noted. In an earlier description of the melanocytes in PET/MCV mice, Nichols and Reams ('60) reported melanocytes occurring in the general body skin of this strain, although the exact location of these cells was not given. In this section, a more detailed description of the occurrence of melanocytes within the skin, as well as in the musculature, of the leg of PET mice will be considered.

Skin

In the present study, leg skin of 5-day postnatal and of adult PET/LSU mice was examined for the presence of melanocytes between the hair follicles. Skin from the anterior and posterior halves of the leg was removed in two large sheets, fixed in 5% formol-saline, dehydrated, and cleared in oil of wintergreen. Skin from the anterior surface of the leg was devoid of pigment cells between the hair follicles, but as the posterior aspect was approached, melanocytes appeared and became more numerous. Skin from the extreme posterior surface possessed an average melanocyte population of 50 to 70 cells per square millimeter, and the population increased toward

the ankle. Sections of posterior leg skin revealed melanocytes to be exclusively dermal in position, and none was seen approaching the dermal-epidermal junction. Adult leg skin retained the distribution and number of melanocytes as seen in the 5-day mouse.

Musculature

The following is a brief description of the muscles consistently pigmented in the leg of PET mice.

Gastrocnemius

The lateral head of the gastrocnemius regularly contained the largest population of melanocytes of all the leg muscles (Fig. 3). In the adult the number of melanocytes reached 1,200, and they were distributed uniformly throughout the muscle. Histological examination of sections of this muscle revealed that melanocytes contributed to the perimysium and endomysium, and would have been indistinguishable from the cells of these connective tissues had it not been for the presence of melanin granules within their cytoplasm. Generally speaking, cytocrine activity was not in evidence, although in some cases melanin granules were seen outside the pigment cells. The medial head of the gastrocnemius had its melanocytes restricted to the distal half of the muscle, and their number reached only one-half to one-third that of the lateral head (400 to 600).

Plantaris

This muscle was rather moderately pigmented, with melanocytes located generally in the distal two-thirds. The number of melanocytes reached 100 in heavily pigmented cases.

Soleus

Melanocytes in this deep muscle were restricted to the distal half, and in no case were pigment cells found in the proximal region. Their number reached 90 in the entire muscle.

Peroneal Group

Per unit area, these four small muscles were the most densely pigmented of all the muscles of the leg, and total melanocyte number attained 500. The anterior edge of this group was less pigmented than the posterior edge, but there was no difference noted in distribution along the proximal-distal axis.

The remaining muscles of the leg rarely possessed pigment cells with the exception of the extensor digitorum longus. In some cases a small number (up to 20) of melanocytes was observed in this muscle, but they seldom were found in the large tibialis anticus on the anterior surface of the leg. In general the posterolateral muscles were more heavily pigmented than the posteromedial ones, and the deeper muscles were seldom pigmented except in the more distal regions. All observed melanocytes in the musculature were rich in melanin and, except for location, could not be distinguished in morphology nor in granule shape from those which occurred in the

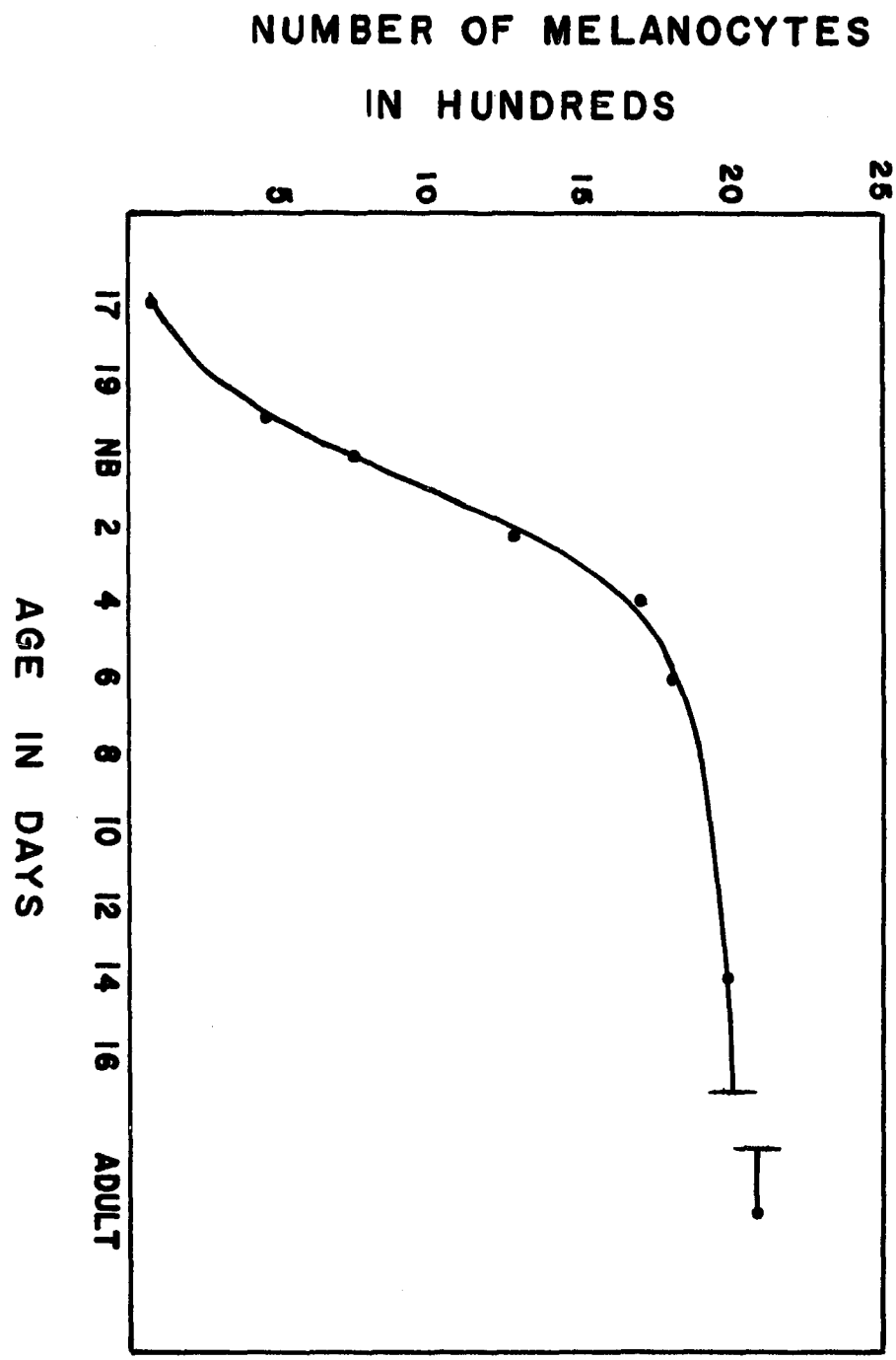
skin (Figs. 4 and 5). Pigment cells in the musculature possessed branches that were usually oriented longitudinally parallel to the muscle fibers, but not infrequently extended across a number of fibers. In these instances melanocytes seemed to be sending processes along the capillaries which coursed transversely through the muscle (Fig. 6). Treatment of entire muscles with DOPA did not increase the number of cells containing dark granules. The average total number of melanocytes in the musculature of the leg of adult PET mouse was 2,100. All the observed cells possessed two to four processes, and the total length of the cells averaged 200 .

Figure 1 shows the rate of increase of the melanocyte population in the musculature of the leg from 17 days of gestation to the adult. Pigment cells first produced melanin in the muscles at day-17. From gestation day-20 to the 5th postnatal day the number of melanocytes increased rapidly and by the end of the first week the adult complement was essentially reached. As the volume of the musculature increased with growth, the melanocyte population density was therefore not maintained, inasmuch as the total pigment cell content remained constant, or showed only slight increase.

In summary, pigmented muscles are found in the posterior and posterolateral areas of the leg, and the skin overlying these areas contains melanocytes between the hair follicles. The distribution of melanocytes in the skin and the musculature thus follows similar correlations. The question arises whether melanoblasts are present

Figure 1

Increase in the total melanocyte population in the leg muscles of PET/LSU mice.


NUMBER OF MELANOCYTES
IN HUNDREDS
5
10
15
20
25
AGE IN DAYS
17
19
NB
2
4
6
8
10
12
14
16
ADULT

uniformly throughout the skin and musculature of the leg, and only differentiate in certain areas such as the posterior region, or whether this pattern is due to a differential migration of melanoblasts into the area. These possibilities will be considered in subsequent sections.

V. MIGRATION OF PIGMENT CELLS INTO THE LEG MUSCULATURE

Since the neural crest is the exclusive source of migratory pigment cells in all vertebrates studied to date, one may ask whether the pigment cells within the musculature of mice also have their origin from the neural crest, and, if so, the manner by which they reach the interior of the leg. To this end, the pigment-forming capacity of mouse tissue from various parts of the leg was tested by grafting it into the coelom of White Leghorn host embryos.

Mesodermal Grafts

In this series donor embryos ranged from 11½ to 17 days of age. Dorsal and ventral surfaces of the limb refer back to the embryonic position during the paddle stage of development. Individual muscles were dissected from embryos 15 days and older. One hundred and thirteen grafts out of 170 operations were recovered from this series. A graft was ruled positive when typical mouse melanocytes were observed within the graft or in the surrounding tissue of the host. In most cases of this type melanocytes were seen to populate a large area of the chick coelom as well as the graft, and were themselves of value in determining the location of the graft. Grafts lacking melanocytes were located by the presence of bone, muscles, or by the unusual vascular supply to the area, and cases were considered negative only if an unquestionable graft was found and melanocytes were lacking.

The earliest embryos tested for melanoblasts in the mesoderm were 11½ days old, and grafts recovered from this group were devoid of melanocytes in all cases. Large masses of muscle attached to well formed bone structures were found occupying a considerable area in the chick coelom. A control of mesoderm with ectoderm was also negative at this age. By 12½ days the control was showing numerous melanocytes, while grafts of both dorsal and ventral mesoderm were unpigmented (Fig. 7). Melanoblast migration had not yet reached the mesoderm. It was only during the 15th day that consistently positive grafts were obtained from the ventral mesoderm of the leg, a full two days after the control grafts of mesoderm with ectoderm were yielding positive results. Thus it appears that during the latter part of the 14th day of development melanoblasts are migrating into the mesoderm from the dermal-epidermal interface, where they had arrived some two days previously. At no time were pigmented grafts regularly obtained from dorsal mesoderm, although a few positive cases were observed at the end of 14 days of development. It is likely that in these instances the dorsal grafts included some tissue that normally would contribute to the peroneal group, and thus would be pigmented. The proliferative and migratory capacity of pigment cells from the gastrocnemius muscle as late as day 17 was shown by the degree of pigmentation of the coelom obtained from 0.2 mm pieces of that tissue (Fig. 8). In no case was a positive graft recovered from grafting portions of tibialis muscle, although 22 examples of this type were obtained for examination.

Ectodermal Grafts

The previous group of experiments shows that the melanocytes found in large numbers associated with the connective tissue of the leg musculature arise from migratory melanoblasts which originate from outside the hind limb itself. Furthermore, the evidence indicates that these melanoblasts migrate along the border between the ectoderm and prospective dermis, and enter the deeper tissues of the leg at later periods of embryonic development. It is the purpose of this group of experiments to investigate more exactly the time sequence of appearance of melanoblasts along the dermal-epidermal junction on both the dorsal and ventral surfaces of the leg. Ninety-three grafts out of 136 operations were recovered from tissue of donor embryos aged 11½ to 13½ days. Ectoderm with the adhering mesodermal cells was stripped from the surface of the leg, divided into 0.2 mm squares, and grafted into the coelom of White Leghorn embryos. Grafts of 11½ day embryos produced skin that was devoid of pigment cells. In all cases the skin formed a spheroidal structure that was easily identified attached to the coelomic wall. In a few instances hair was seen growing toward the center of the graft, but generally the grafts were not permitted to grow a sufficient length of time for well developed hair to form. By the end of the 12th day, grafts from the dorsal surface of the leg were consistently showing the presence of melanoblasts in the 16 cases examined (Fig. 9). However, grafts from ventral skin were negative at this age (Fig. 10), indicating that melanoblasts had not yet

arrived in this region. By 13 days of age grafts from both the dorsal and ventral surface of the leg consistently yielded pigment cells in large numbers. It is noteworthy that extensive migration of melanoblasts from the graft to the peritoneum of the host occurred in this group of experiments as well as in the previous series. The results of both groups of grafting experiments are summarized in Table I.

It is now possible to trace the migration of these pigment cells from their origin in the neural crest to their definitive location within the leg musculature. From the neural crest a wave of proliferating melanoblasts spreads in a medio-lateral direction and reaches the developing limb when the embryo is 11½ to 12 days of age. Within the limb the melanoblasts follow the inner surface of the ectoderm as they populate first the dorsal surface of the leg, and later spread to the ventral surface. Melanoblasts are distributed uniformly over the entire surface of the leg by 13 days, but it is not until the embryo attains an age of 14½ to 15 days that melanoblasts are found within the mesoderm of the ventral surface. At this time the definitive adult pigmentary pattern is established. This migratory sequence is illustrated in Figure 2. In A, embryonic leg skin is represented as a sheet that has been cut along the mid-ventral line of the leg, and spread out flat. The arrows indicate melanoblast migration. In B, a cross section of the leg is shown illustrating the migration of melanoblasts circumferentially to the ventral surface, and then into the mesoderm.

Table I. Production of melanocytes by grafts of PET mouse embryo tissue in the coelom of host chick embryos

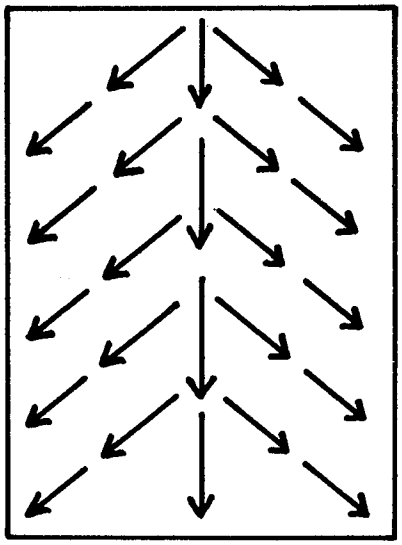
Age in days	Mesodermal Grafts				Ectodermal Grafts			
	Dorsal		Ventral		Dorsal		Ventral	
	+	-	+	-	+	-	+	-
11½		9		9		9		9
12		9		7	7			6
12½		3		4	6		3	9
13					9		10	
13½		5		4	8		11	
14	2	7	1	7				
14½		5	6					
15								
15½		6	7		6			
16								
16½		6*	5**					
17		5*	6**					

* Grafts obtained from transplanting pieces of tibialis muscle.

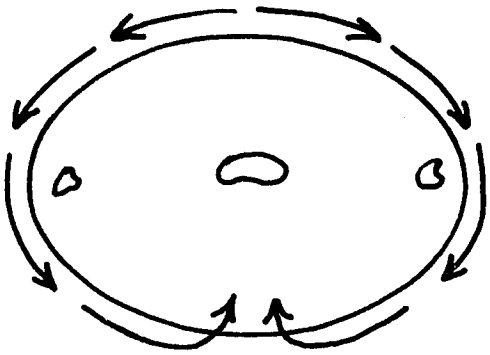
** Grafts obtained from transplanting pieces of gastrocnemius muscle.

Figure 2

Scheme depicting the migratory sequence of melanoblasts into the leg. See text for explanation.



A



B

VI. FACTORS RESPONSIBLE FOR THE PRESENCE OF MELANOCYTES IN THE MUSCULATURE

From the foregoing results it appears that melanoblasts of neural crest origin migrate into the musculature of PET mice, where they differentiate into melanocytes. In this section an examination of the musculature of other mouse strains, and skin grafting experiments were made in an attempt to analyse the factors involved in the occurrence of pigment cells within this extra-epidermal location.

Comparative Study

It is possible that PET mice differ from other strains by possessing within the musculature a melanogenic stimulus necessary for the synthesis of melanin. The tissue environment therefore would be the primary factor involved in the appearance of melanocytes in this extra-epidermal location, and not the presence or absence of pigment cells themselves. On the other hand, if PET mice differ from other strains only in the number of pigment cells, close examination may reveal varying degrees of pigmentation within the leg musculature in all pigmented mice. To test these possibilities, mice of various strains were obtained for study from the Texas Inbred Mice Company, Houston, Texas, and from the Roscoe B.

Jackson Memorial Laboratory, Bar Harbor, Maine. All specimens examined were 5 days of age. In addition, adults of the C57BL strain were studied also. Mice were prepared for examination as described in a previous section. All the pigmented mice examined were found to possess melanocytes within the musculature of the leg (Table II) in the same pattern as that found in the PET strain, although the soleus muscle varied in some forms with regard to the presence of pigment cells. In C57BL mice, for example, muscles always possessing melanocytes were the gastrocnemius, plantaris, soleus and peroneal group. The number of melanocytes within the hind limb muscles was essentially maintained in the adult, as an examination of C57BL mice revealed. The degree of pigmentation within the musculature varied among the different strains studied, but never reached the extent found in the PET mouse. These extra-epidermal melanocytes in all the pigmented mice represented rather conspicuous elements in cleared whole mount preparations of the individual muscles (Fig. 11). The form and color of granule, and the morphology of the melanocytes within the musculature conformed to the genotype of the animal. For example, the leaden genotypes (C57L, DBA) possessed melanocytes of the nucleopetal (enlarged perikaryon, few branches) morphology (Fig. 12), whereas the nucleofugal (highly branched) morphology was visible in the musculature of C57BR and other strains (Fig. 13). The muscle environment apparently has no influence on the granule shape, color or melanocyte morphology.

Table II. Relative numbers of melanocytes in the leg musculature of mice

Strain	Coat Color	Melanocytes
PET	Black	+++
C58	Black	++
DBA/1	Dilute Brown	+
DBA/2	Dilute Brown	+
C3H	Black	++
C57BL	Black	++
C57L	Lead	++
C57BR	Brown	++
SWR	White	0
A	White	0
AKR	White	0
BALB/c	White	0

It is clear from the above facts that melanocytes are found not only in the musculature of PET mice, but are normally occurring components in the hind limb muscles of pigmented mice in general. It would seem that the PET mouse musculature is not unique as was formerly believed with regard to melanoblast differentiation, and that the environment within the musculature of mice in general is capable of supporting melanogenesis, should melanoblasts be present. In no case were melanocytes observed within the musculature of albino mice, although it is possible that amelanotic melanocytes are present but difficult to demonstrate. Further studies are necessary to clarify this point.

Skin Grafts

It appears from the previous observations that the musculature of pigmented mice is able to support melanogenesis. It remains a possibility, however, that the unusually large number of melanocytes in the PET musculature may be due to the availability of a melanogenic substance or chromogen, which may be present to a smaller extent in the musculature of the other pigmented strains. The diffusion of this substance from a higher concentration in the posterior region of the leg to the more anterior regions could likewise account for the observed distribution of melanocytes in the musculature of the PET strain. Thus melanoblasts may be more widespread extra-epidermally than the appearance of melanocytes would

indicate. To investigate this possibility, a series of skin transplantations was performed. Since melanoblasts are known to migrate from the posterior leg skin into the underlying musculature where they produce melanin, it was considered possible that a similar migration may occur into the abdominal musculature from a piece of leg skin transplanted to the belly region. Normally in the PET/LSU strain the abdominal musculature lacks melanocytes, as does the belly skin between the hair follicles. If melanocytes are found in the abdominal muscles as a result of the skin grafting, then this area is established as one supporting melanogenesis. Transplantations of this type may also yield information relating to the cause of the regional differences observed in the occurrence of melanocytes in the skin of the posterior leg and belly of PET mice.

Newborn mice were used for the exchanges in an autoplastic series of grafting experiments. In this group, a 2.0 mm square of belly skin was interchanged with a similar sized piece of skin from the posterior surface of the leg. The mice were permitted to grow for 6 weeks, at which time the grafts were recovered for study. In a homoplastic series, fetal mice of 16 days age served as donors, and newborn mice were recipients of the grafts. Fetal posterior leg skin was grafted to the belly region of one group of hosts, and donor belly skin was grafted to the posterior surface of the leg of another group.

Twenty-five individuals containing autoplastic grafts were recovered for study, and in every instance the abdominal masculature remained free of melanocytes. The same result was obtained in the

homoplastic series. A close examination of the leg skin grafts to the belly revealed the melanocyte content of the grafts to be characteristic of normal posterior leg skin, although it had grown in this heterotopic location for six weeks. In fact, melanocytes were observed in considerable numbers between the hair follicles of the belly skin surrounding the grafts, a region where melanocytes are rarely encountered normally (Fig. 14 and 15). Although the primary purpose of this grafting study was not fulfilled, the migration of pigment cells from the grafts into the belly skin where they appeared as melanocytes between the hair follicles is significant, and indicated that this area is capable of supporting melanogenesis. The reciprocal grafts of belly skin to the posterior leg region maintained the characteristics of normal belly skin, although completely surrounded by the favorable environment of the normal leg skin. In these cases no migration occurred from the leg skin into the melanocyte-free region of the graft, and thus the junction between the graft and the normal leg skin remained quite distinct (Fig. 16). The absence of migration of pigment cells in this case contrasts with the results obtained in the previous grafts of leg skin to the belly, and requires some explanation. It is known that skin undergoes considerable reorganization when it is grafted to another region, or simply reoriented in its normal location (Rawles, '55). In the example of grafting leg skin to the belly, the graft undergoes reorganization with the resulting release of melanoblasts to the

surrounding area. In the reciprocal graft of belly skin to the leg, the graft which possesses relatively few pigment cells is reorganized, while the pigment cell rich leg skin remains stable and withholds its melanoblasts. Therefore no migration is noted in these cases.

The results presented in this section suggest that the pigmentary differences which exist between the regions examined in the PET mouse are the result of differential numbers of pigment cells present in the area, and are not due to some melanogenic substance or stimulus.

The melanocyte content of the leg musculature underlying the belly grafts is of interest in providing information relating to the final stages of melanocyte colonization of the musculature. Since the number of melanocytes in the musculature of PET mice shows a constant increase up to one week after birth, it was considered possible that this increase might be due to a continuous migration of melanoblasts until the final complement is reached. The alternative possibility was that the initial migration of melanoblasts into this region supplied through proliferation the adult number of pigment cells that appeared. The results of the grafting studies indicate that no migration of melanoblasts into the musculature continues after birth, for in all the operated cases the leg musculature was normal in all respects regarding melanocyte content. The large increase in melanocyte numbers after birth is no doubt due to the proliferation of melanoblasts already present within the musculature.

VII. DISCUSSION

Migration of Melanoblasts into the Hind Limb

The results of this investigation are essentially similar to those in the study by Rawles ('47) regarding the first appearance of melanoblasts in the hind limb bud. However, the present investigation deals with the subsequent dispersal and fate of melanoblasts in the hind limb skin and subjacent mesoderm during later stages, and is thus a logical continuation and expansion of her study. By grafting ectoderm with the underlying mesoderm to the coelom of the chick, Rawles was able to demonstrate the presence of prospective pigment cells in the dorsal surface of the limb of C57BL mouse embryos by the 12th day of development. Rawles did not report on ventral skin of this age. Fox ('49), however, made a comparison of the dorsal and ventral surface of the wing and leg bud of Barred Plymouth Rock embryos and found that the dorsal surface receives melanoblasts considerably earlier than the ventral surface. In the chick limb bud the dorsal surface contains melanoblasts by somite stage 43, while in the ventral surface they appear first by somite stage 48. The origin of the extra-epidermal pigment cells in the PET strain is clearly from migratory cells having their origin outside the hind limb. On their passage into the musculature,

melanoblasts first were found colonizing the adjacent skin, and only later move into the mesoderm. Since Rawles has shown that these original skin melanoblasts are of neural crest origin, and since the melanoblasts in the musculature have been shown to migrate from the skin, one is compelled to consider these extra-epidermal pigment cells to be of neural crest origin.

Available evidence indicates that the prospective pigment cells on their journey into the leg bud from the neural crest follow the interior surface of the ectoderm, rather than passing into the limb deep within the mesodermal tissue. In the case of the PET mouse the second route would be more direct for pigment cells to reach their final location within the musculature. However, melanoblasts remain adhering to the inner surface of the skin ectoderm until a relatively late age, when they migrate into the mesodermal tissue. Rawles ('53) has suggested that the pigment cell precursors migrate into the mesenchymal tissue subjacent to the skin ectoderm, and seem to be directed in their movement by the interface between the ectoderm and prospective dermis. Thus removal of the ectoderm of the limb with its few adhering mesodermal cells eliminates the melanoblasts from the limb. In contrast to this view is the claim by some investigators that the migrating melanoblasts are first found in the mesoderm and must move through the differentiating dermis to reach the epidermis (Markert and Silvers, '56). Danneel and Cleffmann ('54) propose that the migrating pigment cells are

present earliest in the dermal-hypodermal junction and migrate later through the dermis into the epidermis. The basis for this claim is the fact that melanoblasts are revealed first in the lower dermis of the snout by silver staining methods in mouse embryos of 14 - 15 days, and only later are they detected in the epidermis. Schumann ('60) also utilized the silver staining method in an effort to analyse the factors involved in the occurrence of the pigment free spot on the forehead of the white blaze strain of mice. Silver-positive cells were found in the skin of the head of embryonic spotted mice by 15 days of age, while in the C57BL strain positive cells were observed in the same region by 13 days. On this evidence it was concluded that in the spotted mice the migration of the neural crest is delayed by two days, and that when melanoblasts reach the head region, the skin is well differentiated and cannot be invaded. It must be pointed out, however, that the silver nitrate staining method reveals melanoblasts at relatively late stages in their maturation into melanocytes, and therefore undifferentiated pigment cells may go undetected. In the present study melanoblasts were found to arrive first in the dorsal surface of the leg, whereas the initial appearance of cells containing melanin granules was in the ventral skin, and not until 16 days of age. It thus seems that the early differentiation of pigment cells in one area is not evidence for an early migration into that area. The staining methods at best detect melanoblasts at relatively late periods in their development. To reveal melanoblasts at their early, highly migratory

stages, one must rely, when possible, on transplantation studies of the type employed in this report.

We can now visualize rather accurately the pattern of movement of the neural crest cells as they first enter the hind limb bud of PET mice. Melanoblasts are found initially along the dorsal surface of the developing limb, and spread distally and circumferentially to the ventral surface. This wave of prospective melanocytes covers the dorsal surface of the limb during the 12th day of development, and one day later they reach the ventral surface of the limb, all the while remaining at the interface between the ectoderm and mesoderm. Two days later melanoblasts appear in the interior tissues of the ventral side of the leg. Melanoblasts never are present in the mesoderm of the dorsal aspect, the interior migration occurring only from ventral skin.

After the pigment cells have first established colonization within the musculature, the question arises as to whether the melanoblasts are capable of proliferation within the muscles of the leg, or whether their population increase is due to a continuous migration until the adult complement is reached. The largest increase in melanocyte number occurs between day-20 of gestation and one week after birth, and at this time spherical DOPA positive cells are observed in large numbers in the skin. At no time are these cells common within the musculature, although on occasion a few may be observed in this location. Reams and Baird ('60) made a study of

the relationship between the morphology and proliferation of pigment cells within the gastrocnemius muscle of PET/MCV mice. Spherical DOPA positive cells were found in large numbers in pieces of the muscle treated, and these cells did not all differentiate into melanocytes at later stages. It was suggested that these DOPA positive cells may contribute to the connective tissue within the muscle. In the present PET/LSU strain this extensive proliferative capacity of melanoblasts within the musculature was found not to exist. It is significant that these muscles are considerably less pigmented than those of the original PET/MCV strain. However, the skin grafting studies have shown that one initial migration of melanoblasts into the interior tissues occurs in PET/LSU mice, and that this initial colonization is sufficient through proliferation to supply all the melanocytes found within the leg musculature of the adult.

Mechanisms Involved in Extra-epidermal Pigmentation

The almost ubiquitous occurrence of melanocytes in mice of the PET strain demonstrates the wide distribution of neural crest cells, for here melanin production serves as a marker indicating their presence. It has been suggested that the distribution of neural crest in other strains of mice, and its contribution to many structures of the body, may be greater than has been appreciated previously (Nichols and Reams, '60). The absence of pigmentation does not necessarily indicate that melanoblasts were unable to reach the

area, but rather that conditions may be unfavorable for melanogenesis in that region. DuShane ('44) has suggested a wider distribution of melanocytes in the fowl embryo than in the adult, and that extra-epidermal structures may produce an inhibitor of melanogenesis, or that pigment cells may die in the extra-epidermal areas. Fox ('49) has studied the distribution of pigment cells in the embryo and hatched Barred Plymouth Rock fowl. She found that prior to hatching melanocytes are widely distributed in many extra-epidermal regions, and that during and immediately after hatching they are lost in some regions or become less numerous. The general coelomic lining, ventral ligament of the gizzard, gonads, and urogenital ducts were the sites of melanoblast infiltration and differentiation as seen by the appearance of melanocytes in these regions by the 14th day of incubation. At hatching their distribution was observed to change, and their number considerably reduced. Even in the adult fowl certain extra-epidermal structures support melanogenesis, especially the gonads, and the unusual distribution of melanocytes in the White Silkie is evidence of the vast dispersal of pigment cells in birds. A similar situation may exist in the mouse (C57BL) in which the superficial epidermis during late gestation and early postnatal life supports melanocytes, only to be lost at later stages when they are confined to the hair follicles. Spotted areas generally are considered not to be the result of differential migration of melanoblasts, but rather due to later factors within the tissue environment (Markert and Silvers, '56; Silvers,

'57). Therefore the fact that differentiated melanocytes are not regularly seen in extra-epidermal regions is not evidence that they were unable to reach the area, or that latent melanoblasts are not present. In fact, in the adult mouse Reynolds ('54) has attempted to demonstrate the presence of amelanotic melanocytes in the superficial epidermis by treating isolated sheets of epidermis with DOPA, silver nitrate, brilliant cresyl blue, and gold impregnation. In some cases the mouse skin was painted with acetone and turpentine, substances known to stimulate melanogenesis through irritation, and then treated with the above reagents. Through these methods Reynolds was able to demonstrate cells which she considered to be inactive melanocytes located throughout the epidermis of the mouse. It must be mentioned, however, that a number of investigators have questioned whether these staining methods actually are specific for pigment cells, as had been believed formerly. In order to investigate the pigment cell content in the skin of the mouse under more natural conditions, the series of auto- and homoplastic grafting experiments were made. In the case of leg skin grafted to the belly of newborn mice, pigment cells migrated from the graft into the belly skin where they were clearly visible as melanocytes located between the hair follicles. This appearance of melanocytes in the belly skin seems to establish the area as one capable of supporting melanogenesis, should melanoblasts be present. Further studies are necessary in order to determine more definitely the pigment cell content of mouse skin.

The factors responsible for these differences in pigmentation between different forms, or within the same individual, have long been discussed, and are of prime importance in an understanding of the observed pigmentary patterns. For example, Markert and Silvers ('56) have examined the occurrence, abundance and morphology of melanocytes in 50 genotypes of mice to determine the relationship between the tissue environment and melanoblast genotype in the differentiation of pigment cells. Their results place the primary responsibility for melanoblast differentiation on the tissue environment. They found that the harderian gland normally produces an environment favorable for melanoblast differentiation. However, mice possessing the genes $Mi^{wh}Mi$ lack the melanogenic stimulus in the harderian gland, and pigment cells are therefore not produced in this region. In the PET mouse similar factors may well operate in determining melanocyte appearance in the extra-epidermal locations, although an alternative possibility is also likely. A few isolated pigment cells are frequently found in the extensor digitorum longus muscle, and their population becomes more dense as one approaches the posterior region of the peroneal group and the gastrocnemius. The deeper muscles are less heavily pigmented than the superficial ones. The impression is that of a differential migration of melanoblasts into the region, with only a few cells reaching the anterior aspect of the leg. Grafts of tibialis muscle at early stages when melanoblasts are entering the

other leg muscles were consistently negative in regard to pigment cell production. In view of the vast migration of pigment cells from grafts of gastrocnemius muscle, one would expect that, were melanoblasts present in the tibialis muscle, some would have been set free to migrate into the favorable host environment and differentiate. The presence of melanocytes in the musculature of pigmented mice in general supports the view that this environment in the PET mouse does not differ basically from that of other mice in which the musculature also supports melanin synthesis. The responsibility for the observed pigmentary pattern in the leg of PET mice therefore appears to rest within the pigment cells themselves, and not due to factors within the tissue environment.

The early migration of melanoblasts into the skin of the leg, and the delay of two days before they penetrate the deeper tissues, suggests an overpopulation phenomenon. Twitty ('44, '53) has shown through in vitro studies in amphibia that pigment cells tend to repel each other, and thus maintain a certain population density per unit area of skin. The extent of migration of pigment cells in culture was dependent upon the size of the entire population. One cell did not migrate, two cells spaced themselves a certain distance apart, and large cultures of melanoblasts exhibited extensive migration. The only difference in migratory behavior noted between Amblystoma and Triturus pigment cells under these conditions was that the extent of outgrowth in Amblystoma was greater than Triturus. Willier ('52) has emphasized the saturation phenomenon in explaining

the spread of melanoblasts throughout the skin in the fowl. He suggested that from their point of origin in the neural crest, a wave of melanoblasts spreads in a dorsal-ventral direction through the skin. Behind the margin of this wave the skin is saturated with melanoblasts, and the cells in this region become fixed or stationary. In front of the spreading wave, an unsaturated condition prevails and melanoblasts are free to move into that region. Willier believed that migration and proliferation of melanoblasts continue until the midventral line is reached, where the two waves meet. At this time the entire skin would be saturated and melanoblast proliferation would cease. Studies in birds have shown that when pigment free skin is grafted to a pigmented host after hatching, melanoblasts migrate freely into the graft and become established in the feather papillae (Rawles, '44). Grafts containing the normal complement of pigment cells do not receive melanoblasts from the pigmented host. In the PET mouse a wave of melanoblasts has been found to pass through the skin of the dorsal surface of the leg during the 12th day of development, and later reach the ventral surface. As the two waves of melanoblasts on both sides of the leg meet ventrally, they do not become stationary, but rather seem to continue their proliferation and invade the musculature. The delay in reaching the interior of the leg suggests a prior saturation of the skin with melanoblasts, after which through continued multiplication they are forced into the deeper tissues. The proliferative and migratory capacity of the PET melanoblasts is

striking when grafts of C57BL mice are compared to those in this report. Even as late as 13½ days PET skin grafts show extensive migration of pigment cells into the host peritoneum, while at earlier stages in the C57BL strain Rawles reported migration was seldom observed. It can be visualized that the great proliferative capacity of PET melanoblasts results in a "spilling over" of these cells from the skin into the extra-epidermal regions of the body. The fact that pigmented mice in general exhibit pigmentation in the hind limb musculature, although to a reduced degree compared to the PET strain, lends support to the above conclusion. It is hardly likely that the pigmentary system in the hind limb skin would be so balanced that only enough melanocytes are produced to populate the skin area alone. In all the pigmented specimens examined, there is an overflow from the skin into the mesoderm, and melanogenesis is thus supported by the mesodermal environment in all these strains. In view of this evidence, one is compelled to consider the pigmentary system in the PET mouse hind limb as differing only quantitatively from that found in other strains of pigmented mice.

SUMMARY

Mice of the PET/LSU strain possess melanocytes consistently within the following muscles of the leg: gastrocnemius, plantaris, soleus, and peroneal group. Melanocytes appear in these locations first by the 17th day of gestation, and the total number increases to approximately 2,100 by one week after birth. This number is maintained in the adult.

Intracoelomic grafting studies have revealed that melanoblasts migrate into the leg along the inner surface ectoderm. They first reach the dorsal surface of the leg by 12 days of age, and by 12½ days melanoblasts are distributed throughout the entire surface of the leg. It is not until 14½ days that melanoblasts are found in the mesoderm on the ventral side, a full two days after they were observed in the skin of this region. The dorsal mesoderm apparently is not invaded by pigment cells.

Pigmented mice of other strains possess melanocytes within the leg musculature, although not in as large a number as in PET mice. Skin grafting experiments indicate that the distribution of melanocytes in PET mice is due to a differential migration of melanoblasts into the area, and not due to a lack of a melanogenic stimulus in certain regions.

SELECTED REFERENCES

Adachi, B. 1903 Hautpigment beim Menschen und bei den Affen. Z. Morph. anthrop., 6: 1-131.

Addison, W.H., and D.Z. Frazer 1932 Variability of pigmentation in the hypophysis and parathyroids of the gray rat (Mus norvegicus). J. Comp. Neurol., 55: 513-523.

Auerbach, R. 1954 Analysis of the developmental effects of a lethal mutation in the house mouse. J. Exp. Zool., 127: 305-330.

Billingham, R.E. 1948 Dendritic cells. J. Anat., Lond., 82: 93-109.

_____, and W.K. Silvers 1960 The melanocytes of mammals. Quart. Rev. Biol., 35: 1-40.

Bischitz, P.G., and R.S. Snell 1959 A study of the melanocytes and melanin in the skin of the male guinea pig. J. Anat., Lond., 93: 233-245.

Burns, R.K. 1939 The differentiation of sex in the opossum (Didelphys virginiana) and its modification by the male hormone testosterone propionate. J. Morph., 65: 79-120.

Danneel, R., and G. Cleffmann 1954 Die Einwanderung der Pigmentzellen in die Haut und die Haare bei Nagetieren. Biol. Zentralbl., 73: 414-428.

Dunn, T.B. 1949 Melanoblasts in the stroma of the parathyroid glands of strain C58 mice. J. Nat. Cancer Inst., 10: 725-733.

DuShane, G. 1944 The embryology of vertebrate pigment cells. Part II. Birds. Quart. Rev. Biol., 19: 98-117.

Fox, M.H. 1949 Analysis of some phases of melanoblast migration in Barred Plymouth Rock embryos. Physiol. Zool., 22: 1-22.

Grant, R. 1933 The pigmentation of the uterine mucosa in the ewe. Vet. J., 89: 271-274.

Gruneberg, H. 1943 The development of some external features in mouse embryos. J. Hered., 34: 88-92.

Markert, C.L., and W.K. Silvers 1956 The effects of genotype and cell environment of melanoblast differentiation in the house mouse. Genetics, 41: 429-450.

Masson, P. 1948 Pigment cells in man. in The Biology of Melanomas. Special publ. N.Y. Acad. Sci., 4: 15-51.

Nichols, S.E., and W.M. Reams, Jr. 1960 The occurrence and morphogenesis of melanocytes in the connective tissues of the PET/MCV mouse strain. J. Embryol. exp. Morph., 8: 24-32.

Rawles, M.E. 1940 The development of melanophores from embryonic mouse tissues grown in the coelom of chick embryos. Proc. Natl. Acad. Sci., Wash., 26: 673-680.

_____. 1944 The migration of melanoblasts after hatching into pigment-free skin grafts of the common fowl. Physiol. Zool., 17: 167-183.

_____. 1947 Origin of pigment cells from the neural crest in the mouse embryo. Physiol. Zool., 20: 248-266.

_____. 1953 Origin of the mammalian pigment cell and its role in the pigmentation of hair. in Pigment Cell Growth. Academic Press. Edited by Myron Gordon. Pp. 1-15.

_____. 1955 Pigmentation in autoplastic and homoplastic grafts of skin from fetal and newborn hooded rats. Am. J. Anat., 97: 79-128.

Reams, W.M., Jr. and S.E. Nichols 1959 The passage and apportionment of pigment cells in the serosa of PET mice. Anat. Record, 133: 326.

_____, and H.R. Baird 1960 The relationship between the morphology and proliferation of pigment cells within the gastrocnemius muscle of PET/MCV mice. Anat. Record, 136: 263.

_____, and T.C. Mayer 1961 Pigment cell behaviour in the hind limb musculature of PET mice. ASB Bull., 8: 26-27.

Reynolds, J. 1954 The epidermal melanocytes of mice. J. Anat., Lond., 88: 45-60.

Schaper, A. 1895 Ueber die sogennanten epithelkorper (Glandulae parathyroideae) in der seitlichen Nachbarschaft der Schildruse und der Umgebung der Arteria carotis der Saeuger und des Menschen. Arch. f. Mikro. Anat., 46: 239-277.

Schumann, H. 1960 Die Entstehung der Scheckung bei Maeusen mit weisser Blesse. Develop. Biol., 2: 505-515.

Silvers, W.K. 1957 A histological and experimental approach to determine the relationship between gold-impregnated dendritic cells and melanocytes. Am. J. Anat., 100: 225-239.

Snell, G.D., E. Fekete, K. Hummel, and L. Law 1940 The relation of mating, ovulation, and the estrous smear in the house mouse to the time of day. Anat. Record, 76: 39-54.

Steiner-Wourlisch, A. 1925 Das melanotische Pigment der Haut bei der grauen Hausmaus (Mus musculus). Z. Zellforsch., 2: 453-479.

Twitty, V.C. 1944 Chromatophore migration as a response to mutual influences of the developing pigment cells. J. exp. Zool., 95: 259-290.

_____. 1953 Intercellular relations in the development of amphibian pigmentation. J. Embryol. exp. Morph., 1: 263-268.

Willier, B.H. 1952 Cells, feathers, and colors. Bios, 23: 109-125.

Zimmermann, A.A., and S.W. Becker 1959 Melanoblasts and melanocytes in fetal negro skin. Illinois Monogr. med. Sci. VI (3).

Figure 3

Unstained whole mount of the lateral head of the gastrocnemius of 5 day old mouse showing melanocyte distribution. X 80.

Plate I

Figure 3

Figure 4

Melanocytes from the soleus muscle of a 5 day old mouse. Unstained whole mount. X 300.

Figure 5

Skin whole mount from a 5 day mouse showing melanocytes in the dermis between the hair follicles. Unstained preparation. X 300.

Plate II

Figure 4

Figure 5

Figure 6

Cross section of the lateral head of the gastrocnemius muscle showing melanocytes between the muscle fibers. Stained with Delafield's hematoxylin. X 800.

Plate III

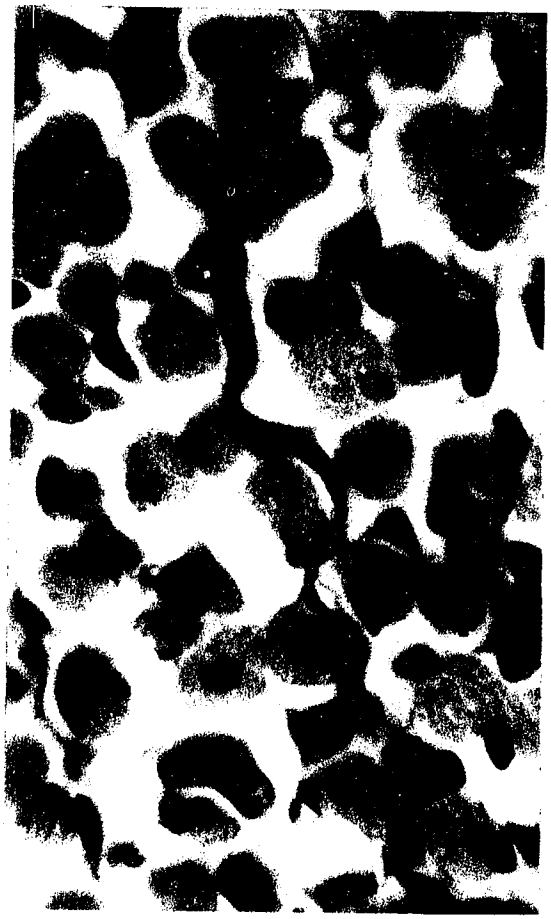


Figure 6

Figure 7

Graft recovered from the transplantation of 12½ day embryonic mouse leg mesoderm to the coelom of the chick. Cartilage and bone are present, but melanocytes are lacking. Unstained whole mount. X 40.

Figure 8

Graft obtained from transplanting 17 day embryonic mouse gastrocnemius to the coelom of the chick. Pigment cells have migrated from the graft into the host peritoneum. Unstained whole mount. X 40.

Plate IV

Figure 7

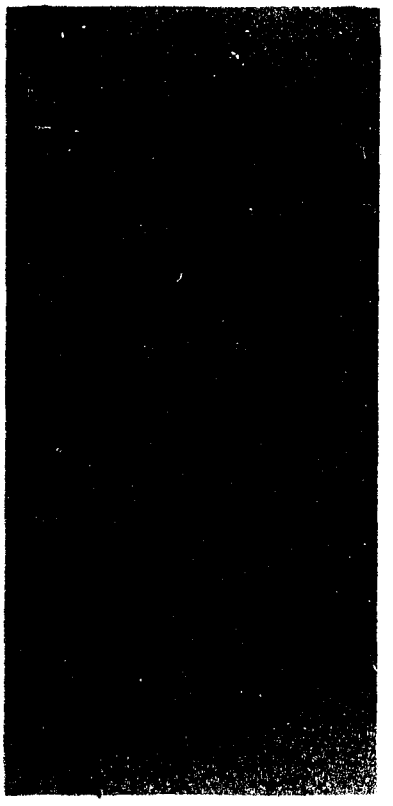


Figure 8

Figure 9

Graft recovered from transplanting ectoderm from the dorsal surface of the leg of a 12 day old mouse embryo to the coelom of the chick. Pigment cells have migrated into the host tissues. Unstained whole mount. X 40.

Figure 10

Graft obtained from transplanting ectoderm from the ventral surface of the leg of a 12 day old mouse embryo to the chick coelom. Pigment cells are absent from this graft. Unstained whole mount. X 40.

Plate V

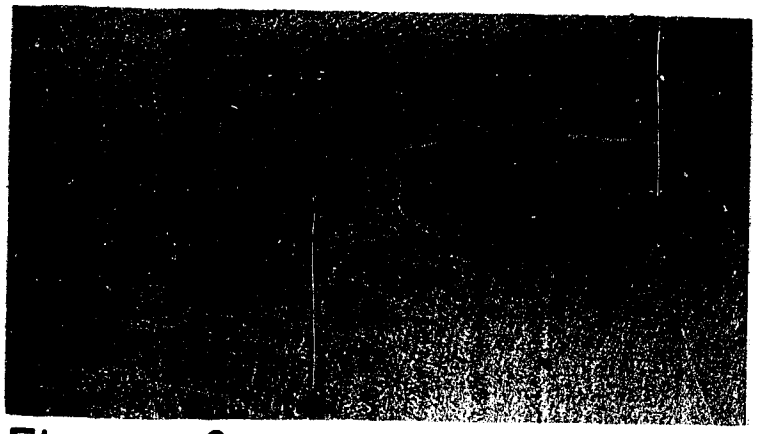


Figure 9

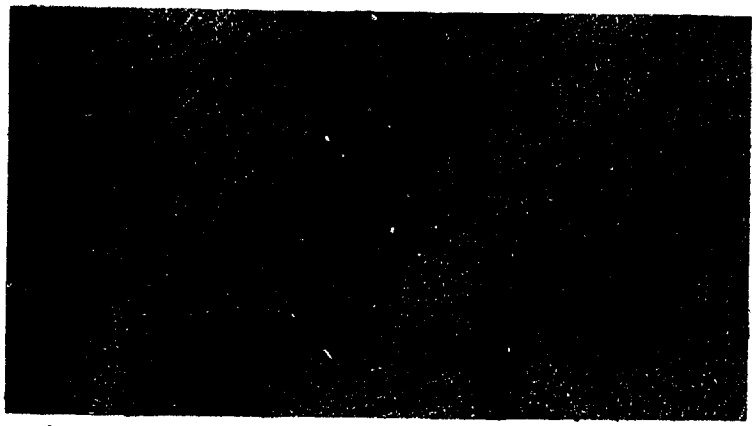


Figure 10

Figure 11

Unstained whole mount of the lateral head of the gastrocnemius of a 5 day old C57BL mouse. X 60.

Figure 12

Unstained melanocyte in the plantaris muscle of a 5 day old C57L mouse. X 300.

Figure 13

Melanocyte in the lateral head of the gastrocnemius muscle of a 5 day old C57BR mouse. Unstained preparation. X 200.

Plate VI

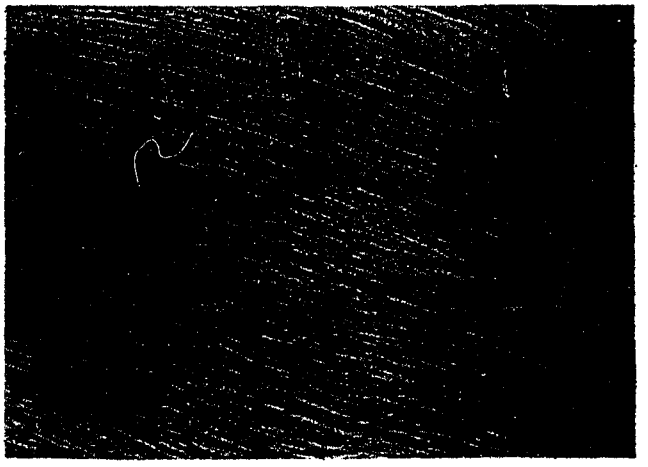


Figure 11

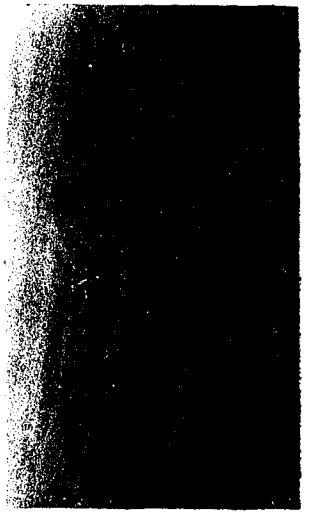


Figure 12

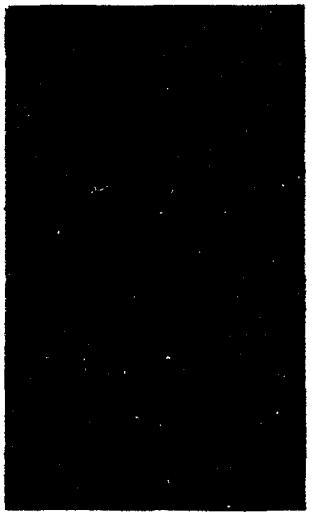


Figure 13

Figure 14

Unstained whole mount of belly skin adjacent to a graft of leg skin. Melanocytes are obvious between the hair follicles. X 35.

Figure 15

Unstained whole mount of normal belly skin. Melanocytes are rare between the hair follicles. X 35.

Plate VII

Figure 14

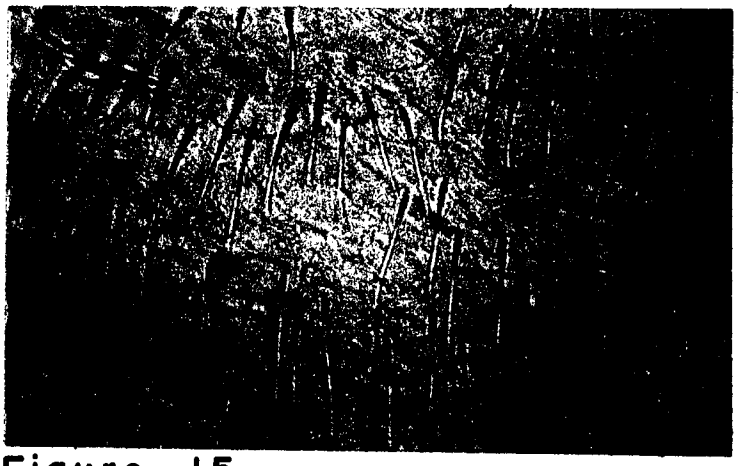


Figure 15

Figure 16

Whole mount showing the junction between normal leg skin (right) and a graft of belly skin (left). Unstained preparation. X 35.

Plate VIII

Figure 16

VITA

Thomas Carl Mayer was born at Pittsburgh, Pennsylvania, on November 30, 1931, and educated through high school in the public schools of Lebanon, Pennsylvania. He was graduated with a B.A. degree in Zoology from the University of Tennessee in 1953, and awarded a Fulbright Fellowship to the University of Tuebingen, Germany, for one year. Mr. Mayer then entered The Johns Hopkins University, supported by a University Scholarship and a Junior Instructorship, and received an M.S. degree in 1957. Following three years of college teaching in the Biological Sciences, Mr. Mayer entered Louisiana State University in the fall of 1960 to work toward the doctorate degree in Zoology. In March, 1961, he was awarded a National Science Foundation Science Faculty Fellowship for a tenure of 15 months.

EXAMINATION AND THESIS REPORT

Candidate: Thomas Carl Mayer

Major Field: Zoology

Title of Thesis: An experimental analysis and description of the melanocytes in the leg of PET mice

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

April 30, 1962